# SPECIFIC DETECTION OF TROPONIN AND MODIFIED FORMS OF TROPONIN

## CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional Application Serial Number 60/536,913, filed January 16, 2004.

## FIELD

[0002] This invention relates to protein biochemistry and clinical diagnostics.

## BACKGROUND

[0003] A diagnostic challenge is to determine the cause of acute chest pain. Typically, these patients arrive at the emergency department with non-specific complaints other than acute chest pain. Although several clinical signs and symptoms can aid in the diagnosis, none can satisfactorily determine the exact cause, which can include (but is not limited to) acute myocardial infarction, angina, aortic dissection, pulmonary embolism, musculoskeletal, myocarditis, Acute coronary syndrome represents the continuum of cardiac pathologies ranging from unstable angina to myocardial infarction. Therefore, several diagnostic tests are typically performed. These include chest radiograph, electrocardiogram, and biomarker testing.

[0004] Three biomarkers are commonly used to aid in the diagnosis of acute coronary syndrome – myoglobin, cardiac troponins, and creatine kinase. Each of these, individually, has specific characteristics that limit its use alone. For example, while myoglobin has a high negative predictive value, it is also poorly specific. The cardiac troponins are thought to be more specific than the myoglobins for myocardial necrosis, but can be absent early during the course of myocardial ischemia. In addition to their diagnostic value, cardiac biomarkers are also being investigated for their prognostic value and their ability to aid in determining the optimal course of therapy.

[0005] Cardiac troponins are used as specific markers for the diagnosis of acute coronary syndrome ("ACS"). Recent studies reported a considerable number of critically ill patients without ACS as being troponin-positive, especially patients with sepsis, pulmonary embolism, renal failure, and stroke. Elevated troponin is a mortality risk factor for medical intensive care patients admitted for reasons other than ACS. It is associated with decreased left ventricular function and higher levels of TNF-alpha and IL-6. Increased cTnT level was the only parameter predicting 15 in-hospital clinical adverse events (*i.e.*, death, thrombolysis, cardiopulmonary resuscitation, and IV use of catecholamine agents).

[0006] Cardiac troponin I (cTnI) is a sensitive marker of cardiac injury, but cTnI assays, like other immunoassays, are susceptible to interferences. Furthermore, the cTnI molecule can exist in phosphorylated, non-phosphorylated, reduced, non-reduced, complexed or non-complexed forms. Each of these forms can change the antigenicity of cTnI, resulting in different antibody-antigen interactions in different experimental formats, thereby giving rise to the disparities in the literature.

[0007] There has been one report on the use of mass spectrometry to study troponin complex, but the mass spectrometric analysis was restricted to peptide mapping of tryptic digest for identification only, there was no report of direct mass spectrometry analysis of the affinity captured complex itself.

## SUMMARY

[0008] Methods are described here for providing isolated, modified forms of troponin, isolated biomolecular interactors with troponin or isolated biomolecular interactors of anti-troponin antibodies that can be found in biological samples.

[0009] In one aspect, a method is described which comprises: capturing polypeptides from a sample. The polypeptides comprise troponin and at least one modified form of troponin. The method further comprises specifically measuring captured troponin. In some further

detailed aspects, the captured troponin is troponin C, troponin I, or troponin T. In some further aspects, the polypeptides are captured with a biospecific capture reagent or a chromatographic adsorbent. In some aspects, the method further comprises measuring a plurality of troponins and determining a relative ratio of amounts, specifically measuring at least one modified form of troponin, or capturing and measuring a polypeptide interactor of troponin, or combinations thereof. In some further aspects, the captured polypeptide is measured by mass spectrometry. In some further aspects, the captured polypeptide is measured by affinity mass spectrometry. In some further aspects, the sample is a subject sample and the method further comprises correlating the detected troponin with a clinical parameter in the subject. In one aspect, the clinical parameter is the presence or absence of acute coronary syndrome.

**[0010]** In another aspect of the invention, a method is described which comprises capturing at least one modified form of troponin polypeptide from a sample. The method further comprises specifically measuring the at least one captured modified form of troponin polypeptide. In some further detailed aspects, the captured troponin is troponin C, troponin I, or troponin T. In some further aspects, the polypeptides are captured with a biospecific capture reagent or a chromatographic adsorbent. In some aspects, the method further comprises capturing and measuring a polypeptide interactor of at least one modified form of troponin. In some further aspects, the captured polypeptide is measured by mass spectrometry. In other further aspects, the captured polypeptide is measured by affinity mass spectrometry. In other further aspects, the sample is a subject sample and the method further comprises correlating the detected modified form of troponin with a clinical parameter in the subject. In one detailed aspect, the clinical parameter is the presence or absence of acute coronary syndrome. In some further aspects, the method further comprises capturing and specifically measuring a plurality of modified forms of troponin from the sample.

**[0011]** In another aspect, a method for discovering polypeptides that interact with troponin is described. The method comprises capturing troponin from a sample with a biospecific capture reagent, removing molecules that are not bound to the biospecific capture reagent or troponin, and measuring molecules bound to the captured troponin. In a further detailed aspect, the molecules are measured by affinity mass spectrometry.

**[0012]** In another aspect of the invention, a method is described which comprises providing a learning set. The learning set comprises a plurality of data objects representing subjects. Each data object comprises data representing a specific measurement of troponin from a subject sample and a clinical parameter of the subject. The method further comprises determining a correlation between the specific measurement of troponin and the clinical

parameters. In some further aspects, providing the learning set comprises the following two steps: (i) capturing troponin from the sample with an antibody, and (ii) specifically measuring captured troponin. In one detailed aspect, the captured polypeptide is measured by affinity mass spectrometry.

**[0013]** In another aspect of the invention, a method is described which comprises providing a learning set. The learning set comprising a plurality of data objects representing subjects. The subjects are classified into a plurality of different clinical parameters. Each data object comprises data representing specific measurement of a plurality of polypeptides from a subject sample. The polypeptides are selected from troponin and at least one modified form of troponin. The method further comprises training a learning algorithm with the learning set, thereby generating a classification model. The classification model classifies a data object according to clinical parameter. In some further aspects, the clinical parameters are selected from presence or absence of disease, risk of disease, stage of disease, response to treatment of disease, and/or class of disease. In some further aspects, the learning set further comprises data representing specific measurement of a polypeptide interactor of troponin. In some further aspects, providing the learning set comprise capturing the polypeptides from the sample with an antibody, and specifically measuring captured polypeptides. In one detailed aspect, the captured polypeptide is measured by affinity mass spectrometry. In some further aspects, the learning algorithm is unsupervised. In other further aspects, the learning algorithm is supervised and each data object further comprises data representing the clinical parameter of the subject. In some further aspects, the method further comprises using the classification model on subject data from a subject of unknown clinical parameter to classify the subject according to a clinical parameter. In some further aspects, the clinical parameter is presence or absence of acute coronary syndrome. In some further aspects, the supervised learning algorithm is selected from linear regression processes, binary decision trees, artificial neural networks, discriminant analyses, logistic classifiers, and support vector classifiers. In other further aspects, the supervised learning algorithm is a recursive partitioning processes.

**[0014]** In another aspect, a method is described which comprises specifically measuring troponin in a subject sample and correlating the measurement with a clinical parameter of the subject. In a detailed aspect, the clinical parameter is acute coronary syndrome. In some further aspects, the method further comprises specifically measuring at least one modified form of troponin and correlating the measurements with the clinical parameter. In some further aspects, the method further comprises specifically measuring at least one biomolecular interactor of troponin or anti-troponin antibody or a modified form of troponin and correlating the

measurement with the clinical parameter. In some further aspects, the method further comprises specifically measuring at least one biomolecular interactor of troponin or anti-troponin antibody or a modified form of troponin and correlating the measurements with the clinical parameter.

**[0015]** In another aspect, a method is described which comprises specifically measuring a modified form of troponin in a subject sample and correlating the measurement with a clinical parameter of the subject. In a detailed aspect, the clinical parameter is acute coronary syndrome. In further aspects, the method further comprises specifically measuring at least one biomolecular interactor of troponin or anti-troponin antibody or a modified form of troponin and correlating the measurements with the clinical parameter.

**[0016]** In another aspect, a method is described which comprises specifically measuring at least one biomolecular interactor of troponin or anti-troponin antibody or a modified form of troponin in a subject sample and correlating the measurements with a clinical parameter of the subject. In a detailed aspect, the clinical parameter is acute coronary syndrome.

**[0017]** In another aspect, a method is described for qualifying an immunoassay calibrator for a troponin immunoassay. The method comprises providing an immunoassay calibrator for a troponin immunoassay. The calibrator comprises a designated concentration of troponin. The method further comprises capturing polypeptides from the calibrator with an anti-troponin antibody and specifically measuring an amount of at least one polypeptide selected from troponin and modified form of troponin captured by the antibody. The measured amount provides an indication of the quality of the immunoassay calibrator. In a detailed aspect, troponin is specifically measured. In another detailed aspect, a modified form of troponin is specifically measured. In another detailed aspect, troponin and a modified form of troponin are specifically measured. In some further aspects, the method comprises determining the amount of troponin captured as a function of total polypeptide captured by the anti-troponin antibody. In some further aspects, the anti-troponin antibody is an antibody used with the immunoassay calibrator in a commercial immunoassay. In a detailed aspect, the amount of troponin captured is measured by affinity mass spectrometry.

**[0018]** In another aspect, a method is described for qualifying an anti-troponin immunoglobulin reagent. The method comprises analyzing an anti-troponin immunoglobulin reagent by mass spectrometry and determining the relative amounts of intact anti-troponin immunoglobulin and anti-troponin immunoglobulin fragments in the reagent.

**[0019]** In another aspect, a method is described which comprises measuring modified forms of an anti-troponin antibody in an antibody reagent for a troponin immunoassay. In some aspects, the method further comprises measuring un-modified forms of the anti-troponin

antibody in the reagent and comparing the measurement of un-modified antibody to the measurement of modified forms of the antibody. In some detailed aspects, the anti-troponin antibody is a monoclonal antibody or a polyclonal antibody. In other detailed aspects, the amount of at least one modified form of troponin in the immunoassay calibration sample is specifically measured. In another detailed aspect, the measurements are performed by affinity mass spectrometry.

**[0020]** In another aspect, a purified modified form of troponin or an interactor of troponin or an interactor of anti-troponin antibody is provided. In some further aspects, the purified modified form of troponin is selected from a splice variant; a post-translational modification, or a product of enzymatic degradation. In a detailed aspect, the purified modified form of troponin is selected from the group consisting of 17455, 10300, 12600, 12900, 9200, and 9320.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0021]** Figure 1 shows specific capture of standard troponin complex added into human serum onto an Anti-TnI antibody coupled on agarose beads.

**[0022]** Figures 2A-2I show mass spectra for specific capture of proteins in patient serum onto an Anti-TnI antibody coupled on agarose beads.

## DETAILED DESCRIPTION

### I. INTRODUCTION

**[0023]** This invention is based in part on the discovery of modified forms of troponin and biomolecular interactors with troponin or anti-troponin antibodies in biological samples. Human troponin exists in three known forms: troponin C, troponin I and troponin T. The amino acid sequences of these proteins are set forth in the Sequence Listing. Allelic forms of each of these also are known.

**[0024]** A protein in a sample can exist in many modified forms. For example, it may be truncated at the amino- or carboxy-terminus through degradation or enzymatic cleavage. Different splice variants of a gene encoding a protein represent modified forms of the protein. Proteins may be subject to post-translational modification including, for example, phosphorylation (adds 80 D per phosphate group), glycosylation, lipidation, methylation (adds 14 D per methyl group), cysteinylation (adds 199 D per cysteinyl group), sulphonation,

glutathionylation (adds 305 D per glutathione group), and acetylation (adds 42 D per acetyl group).

**[0025]** Most immunoassays directed against a particular protein cannot distinguish between different forms of a protein, unless the modification interferes with an epitope recognized by the antibody or antibodies used in the immunoassay. Furthermore, target analytes may be associated with other proteins in a sample with which they interact. Still further, an antibody may bind to molecules in a sample other than the target or modified forms of it. An immunoassay against a target protein typically cannot distinguish these forms or provide detection of proteins that are bound to the target protein.

**[0026]** Mass spectrometry provides a means to specifically detect different forms of a protein and protein interactors in a sample. In mass spectrometry analytes are separated by mass and can be distinguished based on their mass signature. Thus, fragments of a protein can be distinguished from a full-length protein. Furthermore, the mass also can indicate the particular location of the fragment within the protein. Other forms of protein decoration, such as phosphorylation, also provide specific mass signatures that can be identified.

**[0027]** The use of affinity mass spectrometry provides an immunoassay in which a target analyte, its modified forms, and biomolecules that interact with these proteins or the antibody all can be specifically distinguished and measured. Affinity mass spectrometry is a method in which analytes are captured onto a solid surface with an affinity reagent, such as an antibody, another biospecific capture reagent or a chromatographic adsorbent, and detected by mass spectrometry through, *e.g.*, laser desorption/ionization from the surface with subsequent detection and differentiation by mass spectrometry.

## II. MODIFIED FORMS OF TROPONIN AND BIOMOLECULAR INTERACTORS

**[0028]** In one aspect, this invention provides isolated, modified forms of troponin, isolated biomolecular interactors with troponin or isolated biomolecular interactors of anti-troponin antibodies that can be found in biological samples. These modified forms and biomolecular interactors were discovered through affinity mass spectrometry in which analytes from a biological sample were captured on a mass spectrometry probe with an anti-troponin antibody, and specifically detected and distinguished by laser desorption/ionization mass spectrometry from the capture surface. The modified forms and interactors are characterized by molecular weight. Further, certain of these forms have been correlated with specific modifications to troponin, such as truncations. Troponin, modified forms of troponin and biomolecular interactors with troponin are set forth in Table 1, below.

TABLE 1

| Mass-to-charge ratio (Peak area ratio) | Molecular identity |
|---|---|
| M18600 (1) | Full length troponin C (18456 Da) |
| M24600 (1.2) | Full length troponin I (23916 + 160 Da) |
| M34500 (2.5) | Full length troponin T (35792 Da +80) |
| M46900 | Putative interactor of troponin |
| M28060 | Putative troponin interactor (Apolipoprotein AI?) |
| M23800, M25400, M17450 | Putative troponin fragment or interactor |
| M13900 | Putative troponin interactor (Transthyretin?) |
| M12900 | Putative troponin interactor (Transthyretin truncated?) |
| M12600, M10300, M9320, M9200, M8170, M7960, M7795, M6880, M6675, M5115, M5050, M4650, M4485, M4170, M4038, M3905, M3455, M3385, M3280, M3025, M2520, M2270, M2225 | Putative troponin fragments or interactors |

**[0029]** The biomarkers of this invention are characterized by their mass-to-charge ratio as determined by mass spectrometry. The mass-to-charge ratio of each biomarker is provided in Table 1 after the "M." Thus, for example, M6675 has a measured mass-to-charge ratio of 6675. The mass-to-charge ratios were determined from mass spectra generated on a Ciphergen Biosystems, Inc. PBS II mass spectrometer. This instrument has a mass accuracy of about +/- 0.15 percent. Additionally, the instrument has a mass resolution of about 400 to 1000 m/dm, where m is mass and dm is the mass spectral peak width at 0.5 peak height. The mass-to-charge ratio of the biomarkers was determined using Biomarker Wizard$^{tm}$ software (Ciphergen Biosystems, Inc.). Biomarker Wizard assigns a mass-to-charge ratio to a biomarker by clustering the mass-to-charge ratios of the same peaks from all the spectra analyzed, as determined by the PBSII, taking the maximum and minimum mass-to-charge-ratio in the cluster, and dividing by two. Accordingly, the masses provided reflect these specifications.

**[0030]** These molecules can be produced in isolated form by, for example, isolation from natural sources or by chemical synthesis using methods well known in the art.

## III. Specific Detection of Troponin, Modified Forms of Troponin and Biomolecular Interactors with Troponin and Anti-Troponin

**[0031]** In one aspect this invention provides methods for specifically measuring troponin, modified forms of troponin and biomolecular interactors of troponin and anti-troponin antibodies. Specifically measuring an analyte, such as troponin, involves detecting the analyte so as to distinguish it from modified forms of the analyte and from biomolecular interactors. The term "measuring" means detecting the presence or absence of an analyte in a sample or quantifying the amount in relative or absolute terms. A relative amount could be, for example, high, medium or low. An absolute amount could reflect the measured strength of a signal or the translation of this signal strength into another quantitative format, such as micrograms/ml. In another aspect, the invention involves comparing the relative ratio of the forms.

**[0032]** The methods involve capturing troponin, modified forms of troponin and/or biomolecular interactors of troponin and anti-troponin antibodies onto a solid substrate. Typically they will be captured using a biospecific capture reagent against troponin such as an antibody and, in particular, an antibody used in an immunoassay. These molecules also can be captured with non-specific methods, such as chromatographic materials. The captured molecules are then specifically detected and distinguished from one another by any appropriate detection means.

**[0033]** The biomarkers of this invention can be detected by any suitable method. Detection paradigms that can be employed to this end include optical methods, electrochemical methods (voltametry and amperometry techniques), atomic force microscopy, and radio frequency methods, *e.g.*, multipolar resonance spectroscopy. Illustrative of optical methods, in addition to microscopy, both confocal and non-confocal, are detection of fluorescence, luminescence, chemiluminescence, absorbance, reflectance, transmittance, and birefringence or refractive index (*e.g.*, surface plasmon resonance, ellipsometry, a resonant mirror method, a grating coupler waveguide method or interferometry).

**[0034]** In one embodiment, a sample is analyzed by means of a biochip. Biochips generally comprise solid substrates and have a generally planar surface, to which a capture reagent (also called an adsorbent or affinity reagent) is attached. Frequently, the surface of a biochip comprises a plurality of addressable locations, each of which has the capture reagent bound there.

**[0035]** Many protein biochips, adapted for the capture of polypeptides, are described in the art. These include, for example, protein biochips produced by Ciphergen Biosystems, Inc. (Fremont, CA), Packard BioScience Company (Meriden, CT), Zyomyx (Hayward, CA), Phylos (Lexington, MA) and Biacore (Uppsala, Sweden). Examples of such protein biochips are

described in the following patents or published patent applications: U.S. Pat. No. 6,225,047; PCT International Publication No. WO 99/51773; U.S. Pat. No. 6,329,209, PCT International Publication No. WO 00/56934 and U.S. Pat. No. 5,242,828.

### A. Detection by Mass Spectrometry

**[0036]** In a preferred embodiment, the biomarkers of this invention are detected by mass spectrometry, a method that employs a mass spectrometer to detect gas phase ions. Examples of mass spectrometers are time-of-flight, magnetic sector, quadrupole filter, ion trap, ion cyclotron resonance, electrostatic sector analyzer and hybrids of these.

**[0037]** In a further preferred method, the mass spectrometer is a laser desorption/ionization mass spectrometer. In laser desorption/ionization mass spectrometry, the analytes are placed on the surface of a mass spectrometry probe, a device adapted to engage a probe interface of the mass spectrometer and to present an analyte to ionizing energy for ionization and introduction into a mass spectrometer. A laser desorption mass spectrometer employs laser energy, typically from an ultraviolet laser, but also from an infrared laser, to desorb analytes from a surface, to volatilize and ionize them and make them available to the ion optics of the mass spectrometer.

#### 1. SELDI

**[0038]** A preferred mass spectrometric technique for use in the invention is "Surface Enhanced Laser Desorption and Ionization" or "SELDI," as described, for example, in U.S. Pat. No. 5,719,060 and No. 6,225,047, both to Hutchens and Yip. This refers to a method of desorption/ionization gas phase ion spectrometry (*e.g.*, mass spectrometry) in which an analyte (here, one or more of the biomarkers) is captured on the surface of a SELDI mass spectrometry probe. There are several versions of SELDI.

**[0039]** One version of SELDI is called "affinity capture mass spectrometry." It also is called "Surface-Enhanced Affinity Capture" or "SEAC". This version involves the use of probes that have a material on the probe surface that captures analytes through a non-covalent affinity interaction (adsorption) between the material and the analyte. The material is variously called an "adsorbent," a "capture reagent," an "affinity reagent" or a "binding moiety." Such probes can be referred to as "affinity capture probes" and as having an "adsorbent surface." The capture reagent can be any material capable of binding an analyte. The capture reagent may be attached directly to the substrate of the selective surface, or the substrate may have a reactive surface that carries a reactive moiety that is capable of binding the capture reagent, *e.g.*, through a reaction forming a covalent or coordinate covalent bond. Epoxide and acyl-imidizole are useful reactive

moieties to covalently bind polypeptide capture reagents such as antibodies or cellular receptors. Nitrilotriacetic acid and iminodiacetic acid are useful reactive moieties that function as chelating agents to bind metal ions that interact non-covalently with histidine containing peptides. Adsorbents are generally classified as chromatographic adsorbents and biospecific adsorbents.

**[0040]** Chromatographic adsorbents include those adsorbent materials typically used in chromatography. Chromatographic adsorbents include, for example, ion exchange materials, metal chelators (*e.g.*, nitrilotriacetic acid or iminodiacetic acid), immobilized metal chelates, hydrophobic interaction adsorbents, hydrophilic interaction adsorbents, dyes, simple biomolecules (*e.g.*, nucleotides, amino acids, simple sugars and fatty acids) and mixed mode adsorbents (*e.g.*, hydrophobic attraction/electrostatic repulsion adsorbents).

**[0041]** Biospecific adsorbents include those molecules that specifically bind to a biomolecule. Typically they comprise a biomolecule, *e.g.*, a nucleic acid molecule (*e.g.*, an aptamer), a polypeptide, a polysaccharide, a lipid, a steroid or a conjugate of these (*e.g.*, a glycoprotein, a lipoprotein, a glycolipid, a nucleic acid (*e.g.*, DNA)-protein conjugate). In certain instances, the biospecific adsorbent can be a macromolecular structure such as a multiprotein complex, a biological membrane or a virus. Examples of biospecific adsorbents are antibodies, receptor proteins and nucleic acids. Biospecific adsorbents typically have higher specificity for a target analyte than chromatographic adsorbents. Further examples of adsorbents for use in SELDI can be found in U.S. Pat. No. 6,225,047. A "bioselective adsorbent" refers to an adsorbent that binds to an analyte with an affinity of at least $10^{-8}$ M.

**[0042]** Protein biochips produced by Ciphergen Biosystems, Inc. comprise surfaces having chromatographic or biospecific adsorbents attached thereto at addressable locations. Ciphergen ProteinChip® arrays include NP20 (hydrophilic); H4 and H50 (hydrophobic); SAX-2, Q-10 and LSAX-30 (anion exchange); WCX-2, CM-10 and LWCX-30 (cation exchange); IMAC-3, IMAC-30 and IMAC 40 (metal chelate); and PS-10, PS-20 (reactive surface with acyl-imidizole, expoxide) and PG-20 (protein G coupled through acyl-imidizole). Hydrophobic ProteinChip® arrays have isopropyl or nonylphenoxy-poly(ethylene glycol)methacrylate functionalities. Anion exchange ProteinChip® arrays have quaternary ammonium functionalities. Cation exchange ProteinChip® arrays have carboxylate functionalities. Immobilized metal chelate ProteinChip® arrays have nitrilotriacetic acid functionalities that adsorb transition metal ions, such as copper, nickel, zinc, and gallium, by chelation. Preactivated ProteinChip® arrays have acyl-imidizole or epoxide functional groups that can react with groups on proteins for covalent binding.

[0043] Such biochips are further described in: Hutchens and Yip, U.S. Pat. No. 6,579,719, 2003; PCT Publication No. WO 00/66265, Rich *et al.*, 2000; U.S. Pat. No. 6,555,813, Beecher *et al.*, 2003; U.S. Pat. Application No. U.S. 2003 0032043 A1, Pohl and Papanu,; and PCT Publication No. WO 03/040700, Um *et al.*, 2003; U.S. Provisional Pat. Application No. 60/367,837, Boschetti *et al.*, filed May 5, 2002; and U.S. Pat. Application No. 60/448,467, Huang *et al.*, filed February 21, 2003.

**[0044]** In general, a probe with an adsorbent surface is contacted with the sample for a period of time sufficient to allow biomarker or biomarkers that may be present in the sample to bind to the adsorbent. After an incubation period, the substrate is washed to remove unbound material. Any suitable washing solutions can be used; preferably, aqueous solutions are employed. The extent to which molecules remain bound can be manipulated by adjusting the stringency of the wash. The elution characteristics of a wash solution can depend, for example, on pH, ionic strength, hydrophobicity, degree of chaotropism, detergent strength, and temperature. Unless the probe has both SEAC and SEND properties (as described herein), an energy absorbing molecule then is applied to the substrate with the bound biomarkers.

**[0045]** The biomarkers bound to the substrates are detected in a gas phase ion spectrometer such as a time-of-flight mass spectrometer. The biomarkers are ionized by an ionization source such as a laser, the generated ions are collected by an ion optic assembly, and then a mass analyzer disperses and analyzes the passing ions. The detector then translates information of the detected ions into mass-to-charge ratios. Detection of a biomarker typically will involve detection of signal intensity. Thus, both the quantity and mass of the biomarker can be determined.

**[0046]** Another version of SELDI is Surface-Enhanced Neat Desorption (SEND), which involves the use of probes comprising energy absorbing molecules that are chemically bound to the probe surface ("SEND probe"). The phrase "energy absorbing molecules" (EAM) denotes molecules that are capable of absorbing energy from a laser desorption/ionization source and, thereafter, contribute to desorption and ionization of analyte molecules in contact therewith. The EAM category includes molecules used in MALDI, frequently referred to as "matrix," and is exemplified by cinnamic acid derivatives, sinapinic acid (SPA), cyano-hydroxy-cinnamic acid (CHCA) and dihydroxybenzoic acid, ferulic acid, and hydroxyaceto-phenone derivatives. In certain embodiments, the energy absorbing molecule is incorporated into a linear or cross-linked polymer, *e.g.*, a polymethacrylate. For example, the composition can be a co-polymer of α-cyano-4-methacryloyloxycinnamic acid and acrylate. In another embodiment, the composition is a co-polymer of α-cyano-4-methacryloyloxycinnamic acid, acrylate and 3-(tri-ethoxy)silyl

propyl methacrylate. In another embodiment, the composition is a co-polymer of α-cyano-4-methacryloyloxycinnamic acid and octadecylmethacrylate ("C18 SEND"). SEND is further described in U.S. Pat. No. 6,124,137 and PCT Publication No. WO 03/64594, Kitagawa, 2003).

**[0047]** SEAC/SEND is a version of SELDI in which both a capture reagent and an energy absorbing molecule are attached to the sample presenting surface. SEAC/SEND probes therefore allow the capture of analytes through affinity capture and ionization/desorption without the need to apply external matrix. The C18 SEND biochip is a version of SEAC/SEND, comprising a C18 moiety which functions as a capture reagent, and a CHCA moiety which functions as an energy absorbing moiety.

**[0048]** Another version of SELDI, called Surface-Enhanced Photolabile Attachment and Release (SEPAR), involves the use of probes having moieties attached to the surface that can covalently bind an analyte, and then release the analyte through breaking a photolabile bond in the moiety after exposure to light, *e.g.*, to laser light (see, U.S. Pat. No. 5,719,060). SEPAR and other forms of SELDI are readily adapted to detecting a biomarker or biomarker profile, pursuant to the present invention.

**2. Other Mass Spectrometry Methods**

**[0049]** In another mass spectrometry method, the biomarkers can be first captured on a chromatographic resin that binds the target molecules. For example, the resin can be derivatized with an anti-troponin antibody. Alternatively, this method could be preceded by fractionating the sample on an anion exchange resin before application to the cation exchange resin. After elution from the resin, the sample can be analyzed by MALDI, electrospray, or another ionization method for mass spectrometry. In another alternative, one could fractionate on an anion exchange resin and detect by MALDI or electrospray mass spectrometry directly. In yet another method, one could capture the biomarkers on an immuno-chromatographic resin that comprises antibodies that bind the biomarkers, wash the resin to remove unbound material, elute the biomarkers from the resin and detect the eluted biomarkers by MALDI, SELDI, electrospray mass spectrometry or another ionization mass sprectrometry method.

**3. Data Analysis**

**[0050]** Analysis of analytes by time-of-flight mass spectrometry generates a time-of-flight spectrum. The time-of-flight spectrum ultimately analyzed typically does not represent the signal from a single pulse of ionizing energy against a sample, but rather the sum of signals from a number of pulses. This reduces noise and increases dynamic range. This time-of-flight data is then subject to data processing. In Ciphergen's ProteinChip® software, data processing typically

includes TOF-to-M/Z transformation to generate a mass spectrum, baseline subtraction to eliminate instrument offsets and high frequency noise filtering to reduce high frequency noise.

**[0051]** Data generated by desorption and detection of biomarkers can be analyzed with the use of a programmable digital computer. The computer program analyzes the data to indicate the number of biomarkers detected, and optionally the strength of the signal and the determined molecular mass for each biomarker detected. Data analysis can include steps of determining signal strength of a biomarker and removing data deviating from a predetermined statistical distribution. For example, the observed peaks can be normalized, by calculating the height of each peak relative to some reference. The reference can be background noise generated by the instrument and chemicals such as the energy absorbing molecule which is set at zero in the scale.

**[0052]** The computer can transform the resulting data into various formats for display. The standard spectrum can be displayed, but in one useful format only the peak height and mass information are retained from the spectrum view, yielding a cleaner image and enabling biomarkers with nearly identical molecular weights to be more easily seen. In another useful format, two or more spectra are compared, conveniently highlighting unique biomarkers and biomarkers that are up- or down-regulated between samples. Using any of these formats, one can readily determine whether a particular biomarker is present in a sample.

**[0053]** Analysis generally involves the identification of peaks in the spectrum that represent signal from an analyte. Peak selection can be done visually, but software is available, as part of Ciphergen's ProteinChip® software package, that can automate the detection of peaks. In general, this software functions by identifying signals having a signal-to-noise ratio above a selected threshold and labeling the mass of the peak at the centroid of the peak signal. In one useful application, many spectra are compared to identify identical peaks present in some selected percentage of the mass spectra. One version of this software clusters all peaks appearing in the various spectra within a defined mass range, and assigns a mass (M/Z) to all the peaks that are near the mid-point of the mass (M/Z) cluster.

**[0054]** Software used to analyze the data can include code that applies an algorithm to the analysis of the signal to determine whether the signal represents a peak in a signal that corresponds to a biomarker according to the present invention. The software also can subject the data regarding observed biomarker peaks to classification tree or ANN analysis, to determine whether a biomarker peak or combination of biomarker peaks is present that indicates the status of the particular clinical parameter under examination. Analysis of the data may be "keyed" to a variety of parameters that are obtained, either directly or indirectly, from the mass spectrometric analysis of the sample. These parameters include, but are not limited to, the presence or absence

or one or more peaks, the shape of a peak or group of peaks, the height of one or more peaks, the log of the height of one or more peaks, and other arithmetic manipulations of peak height data.

#### 4. General protocol for SELDI detection of troponin, modified forms and interactors

**[0055]** A preferred protocol for the detection of the biomarkers of this invention is as follows. The biological sample to be tested as used herein is a sample of biological tissue or fluid and includes human and animal body fluid such as whole blood, plasma, white blood cells, cerebrospinal fluid, urine, semen, vaginal secretions, lymphatic fluid, and various external secretions of the respiratory, intestinal and genitourinary tracts, tears, saliva, milk, ductal lavage, seminal plasma, tissue biopsy, fixed tissue specimens, fixed cell specimens, cell extracts and cell culture supernatents and derivatives of these, *e.g.*, blood or a blood derivative such as serum, preferably is subject to pre-fractionation before SELDI analysis. This simplifies the sample and improves sensitivity. A preferred method of pre-fractionation involves contacting the sample with an anion exchange chromatographic material, such as Q HyperD (BioSepra, SA). The bound materials are then subject to stepwise pH elution using buffers at pH 9, pH 7, pH 5 and pH 4Various fractions containing the biomarker are collected.

The sample to be tested (preferably pre-fractionated) is then contacted with an affinity capture probe comprising an anti-troponin antibody, *e.g.*, a pre-activated PS10 or PS20 ProteinChip® array (Ciphergen Biosystems, Inc.). The probe is washed with a buffer that will retain troponin, modified forms of troponin and/or biomolecular interactors of troponin and anti-troponin antibodies while washing away unbound molecules. A suitable wash for these molecules is the buffer identified in the Example. The analytes are detected by laser desorption/ionization mass spectrometry.

### B. Detection by Immunoassays

**[0056]** In another embodiment, the biomarkers of this invention can be measured by immunoassay. Immunoassay requires biospecific capture reagents, such as antibodies, to capture the biomarkers. Antibodies can be produced by methods well known in the art, *e.g.*, by immunizing animals with the biomarkers. Biomarkers can be isolated from samples based on their binding characteristics. Alternatively, if the amino acid sequence of a polypeptide biomarker is known, the polypeptide can be synthesized and used to generate antibodies by methods well known in the art.

**[0057]** This invention contemplates traditional immunoassays including, for example, sandwich immunoassays including ELISA or fluorescence-based immunoassays, as well as other enzyme immunoassays. In the SELDI-based immunoassay, a biospecific capture reagent for the

biomarker is attached to the surface of an MS probe, such as a pre-activated ProteinChip® array. The biomarker is then specifically captured on the biochip through this reagent, and the captured biomarker is detected by mass spectrometry.

**[0058]** Biospecific adsorbents include those molecules that bind a target analyte with an affinity of at least $10^{-9}$ M, $10^{-10}$ M, $10^{-11}$ M or $10^{-12}$ M. As is well understood in the art, biospecific capture reagents include antibodies, binding fragments of antibodies (*e.g.*, single chain antibodies, Fab' fragments, F(ab)'2 fragments, and scFv proteins and affibodies (Affibody, Teknikringen 30, floor 6, Box 700 04, Stockholm SE-10044, Sweden, U.S. Pat. No.: 5,831,012)). Depending on intended use, they also may include receptors and other proteins that specifically bind another biomolecule.

## IV. Correlation of a Specific Measurement of Troponin Forms and Interactors with Specific Clinical Parameters

### A. Clinical Diagnostics

A principle of diagnostic testing is the correlation of the results of a procedure (*e.g.*, blood test, urine test, CSF, test, sputum test, tissue biopsy, radiologic examination, measurement of one or more biomarkers, and the like) with particular clinical parameters. The correlation necessarily involves a comparison between two or more groups distinguished by the clinical parameter. A clinical parameter could be, for example, presence or absence of disease, risk of disease, stage of disease, severity of disease, class of disease or response to treatment of disease. Accordingly, the diagnostician uses this correlation to qualify the status of a subject with respect to the clinical parameter. That is, the diagnostician uses the results of a procedure on a subject to classify or diagnose a subject status with respect to a clinical parameter, the confidence of the diagnosis/classification being related to the classifying or splitting power of the signs or symptoms used in the test.

**[0059]** Biomarkers having the most diagnostic utility, such as those of this invention, show a statistical difference in different clinical parameters of at least $p \leq 0.05$, $p \leq 10^{-2}$, $p \leq 10^{-3}$, $p \leq 10^{-4}$ or $p \leq 10^{-5}$. Diagnostic tests that use these biomarkers alone or in combination show a sensitivity and specificity of at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% and about 100%.

**[0060]** The power of a diagnostic test to correctly predict status is commonly measured as the sensitivity of the assay, the specificity of the assay or the area under a receiver operated characteristic ("ROC") curve. Sensitivity is the percentage of true positives that are predicted by a test to be positive, while specificity is the percentage of true negatives that are predicted by a test to be negative. An ROC curve provides the sensitivity of a test as a function of 1-specificity.

The greater the area under the ROC curve, the more powerful the predictive value of the test. Other useful measures of the utility of a test are positive predictive value and negative predictive value. Positive predictive value is the percentage of actual positives that test as positive. Negative predictive value is the percentage of actual negatives that test as negative.

**[0061]** In the present case, elevated levels of troponin correlate with heart disease, more particularly cardiac tissue damage and acute cardiac syndrome. The diagnostician can use a measurement of troponin to qualify the heart disease status of a subject. For example, a doctor can use the amount of troponin in a patient blood sample to diagnose the presence or absence of acute coronary syndrome. The phrase "acute coronary syndrome status" includes distinguishing, *inter alia*, acute coronary syndrome v. non-acute coronary syndrome. Troponin levels may also be used to determine respiratory status in patients (respiratory syncytial virus (*Am J Emerg Med.* **21**(6):479-82, 2003), pulmonary embolism (*Curr Opin Pulm Med.* **9**(5):374-7), 2003; pulmonary hypertension (*Circulation* **108**(7):844-8, 2003; myopericarditis, blunt trauma with or without cardiac contusion, cardiac involvement of systemic disease (*e.g.* cardiac sarcoidosis), rejection of cardiac transplant, cardiac valvular disease, arrythmias, heart failure due to, as examples, hypertrophic or dilated cardiomyopathies, congenital and/or acquired cardiomyopathies, acute and/or chronic renal failure, monitoring of response or toxicity to pharmacologic therapy (*e.g.*, fibrinolytic agents).

**[0062]** A typical troponin immunoassay does not distinguish between troponin and modified forms of troponin captured by the antibody, and also does not detect protein interactors. Therefore, the typical immunoassay results in the correlation of all troponin forms together with the clinical parameter of interest, *e.g.*, acute coronary syndrome. However, by specifically distinguishing the measurements of troponin, its various forms and interactors, this invention allows the specific correlation of these analytes with the clinical parameter. Specific correlation of particular analytes in a sample provides greater specificity and sensitivity in diagnosis.

**[0063]** The following are recommended for meaningful troponin assays: They should use antibodies that recognize epitopes not affected by proteolysis; should react with post-translationally modified troponins; should be standardized between manufacturers using internationally accepted standards when they become available; should be free of HAMA, RF, fibrin and other interferences.

**B. Use of specific measurement of troponin and its forms in determining clinical status of patients**

**[0064]** Accordingly, in one aspect this invention provides diagnostic, prognostic and theranostic methods using the specific measurement of at least one biomarker selected from

troponin, modified forms of troponin or biomolecular interactors of troponin and anti-troponin antibodies with these molecules, *e.g.*, those identified in Table 1. The methods involve first providing a specific measurement of the target form of troponin by any method, and then correlating the measurement with the clinical parameter of interest, *e.g.*, acute coronary syndrome. By correlating the measurement, one is able to qualify the subject status with respect to the particular clinical parameter in question. Based on this correlation, further procedures may be indicated, including additional diagnostic tests or therapeutic procedures or regimens. Each of the biomarkers of this invention can be individually correlated with disease.

**[0065]** Any form of troponin or protein interactor, individually, is useful in aiding in the determination of acute coronary syndrome status. First, the selected biomarker is specifically measured in a subject sample using the methods described herein, *e.g.*, capture on a SELDI biochip followed by detection by mass spectrometry. Then, the measurement is compared with a diagnostic amount or cutoff that distinguishes one diagnostic parameter from another, *e.g.*, a positive acute coronary syndrome status from a negative acute coronary syndrome status. The diagnostic amount represents a measured amount of a biomarker above which or below which a subject is classified as having a particular clinical parameter. For example, if the biomarker is up-regulated compared to normal in clinical parameter, then a measured amount above the diagnostic cutoff provides a diagnosis of clinical parameter. Alternatively, if the biomarker is down-regulated in acute coronary syndrome, then a measured amount below the diagnostic cutoff provides a diagnosis of acute coronary syndrome. As is well understood in the art, by adjusting the particular diagnostic cutoff used in an assay one can increase sensitivity or specificity of the diagnostic assay depending on the preference of the diagnostician.

**[0066]** In some embodiments, the mere presence or absence of a biomarker, without quantifying the amount of the biomarker, is useful and can be correlated with a probable diagnosis of acute coronary syndrome. Thus, a detected presence or absence, respectively, of these markers in a subject being tested indicates that the subject has a higher probability of having acute coronary syndrome.

**[0067]** While individual biomarkers are useful diagnostic markers, it has been found that a combination of biomarkers can provide greater predictive value of a particular status than single markers alone. Specifically, the detection of a plurality of markers in a sample can increase the percentage of true positive and true negative diagnoses and decreases the percentage of false positive or false negative diagnoses. Thus, in one embodiment, one measures the relative ratio of various forms of troponin, modified forms of troponin or troponin interactors.

[0068] In certain embodiments of the methods of qualifying acute coronary syndrome status, the methods further comprise managing subject treatment based on the status. Such management describes the actions of the physician or clinician subsequent to determining acute coronary syndrome status. For example, if a physician makes a diagnosis of acute coronary syndrome, then a certain regime of treatment, such as medical intervention (*e.g.*, statins, beta blocker, glycoprotein IIb/IIIa inhibitor) or invasive intervention (*e.g.*, revascularization) might follow. The specific complement of biomarkers and their interactors can predict the optimal course of treatment. Alternatively, a diagnosis of non-acute coronary syndrome might be followed with no treatment. If the diagnostic test gives an inconclusive result on acute coronary syndrome status, further tests may be called for.

## V. DISCOVERY OF PATTERNS OF TROPONIN FORMS CORRELATED WITH CLINICAL PARAMETERS

[0069] While single target analytes have traditionally been used as correlates of clinical parameters, such as presence or absence of disease, scientists and physicians have taken increasing interest in the use of multiple makers. This approach has become possible as a result of new technologies, such as gene arrays and affinity mass spectrometry that allow differential detection of many different molecules in a clinical sample. The discovery of patterns of molecules that can be correlated with a clinical parameter involves the multivariate analysis of measurements of a plurality of molecules, such as proteins, in a sample.

[0070] Accordingly, in one aspect this invention provides a method for discovering patterns of proteins including troponin, modified forms of troponin or biomolecules, *e.g.*,, those identified in Table 1, that interact with these, which patterns correlate with a clinical parameter of interest. This method involves training a learning algorithm with a learning set of data that includes measurements of the aforementioned molecules and generating a classification algorithm that can classify an unknown sample into a class represented by clinical parameter.

[0071] The method involves, first, providing a learning set of data.. The learning set includes data objects. Each data object represents a subject for which clinical data has been developed. The clinical data included in the data object includes the specific measurements of troponin, modified forms of troponin and biomolecular interactors of troponin and anti-troponin antibodies with these. Each subject is classified into one of at least two different clinical parameter classes. For example, the clinical parameters could include presence or absence of disease, risk of disease, stage of disease, response to treatment of disease or class of disease.

[0072] In a preferred embodiment, the learning set will be in the form of a table in which, for example, each row is data object representing a sample. The columns contain

information identifying the subject, data providing the specific measurements of each of the molecules measured and optionally identifying the clinical parameter associated with the subject.

**[0073]** The learning set is then used to train a classification algorithm. Classification models can be formed using any suitable statistical classification (or "learning") method that attempts to segregate bodies of data into classes based on objective parameters present in the data. Classification methods may be either supervised or unsupervised. Examples of supervised and unsupervised classification processes are described in Jain, "Statistical Pattern Recognition: A Review", IEEE Transactions on Pattern Analysis and Machine Intelligence, Vol. 22, No. 1, January 2000.

**[0074]** In supervised classification, each data object includes data indicating the clinical parameter class to which the subject belongs. Examples of supervised classification processes include linear regression processes (*e.g.*, multiple linear regression (MLR), partial least squares (PLS) regression and principal components regression (PCR)), binary decision trees (*e.g.*, recursive partitioning processes such as CART - classification and regression trees), artificial neural networks such as back propagation networks, discriminant analyses (*e.g.*, Bayesian classifier or Fischer analysis), logistic classifiers, and support vector classifiers (support vector machines). A preferred supervised classification method is a recursive partitioning process. Recursive partitioning processes use recursive partitioning trees to classify spectra derived from unknown samples.

**[0075]** In other embodiments, the classification models that are created can be formed using unsupervised learning methods. Unsupervised classification attempts to learn classifications based on similarities in the training data set. In this case, the data representing the class to which the subject belongs is not included in the data object representing that subject, or such data is not used in the analysis. Unsupervised learning methods include cluster analyses. Clustering techniques include the MacQueen's K-means algorithm and the Kohonen's Self-Organizing Map algorithm.

**[0076]** Learning algorithms asserted for use in classifying biological information are described, for example, in PCT Publication No. WO 01/31580, Barnhill *et al.*, "Methods and devices for identifying patterns in biological systems and methods of use thereof"), U.S. Pat. Application 2002 0193950 A1, Gavin *et al.*; U.S. Pat. Application 2003 0004402 A1, Hitt *et al.*; and U.S. Pat. Application 2003 0055615 A1, Zhang and Zhang,.

[0077] Thus trained, learning algorithm will generate a classification model that classifies a sample into one of the classification groups. The classification model usually involves a subset of all the markers included in the learning set. The classification model can be used to classify an unknown sample into one of the groups.

## VI. Determining the Quality of an Immunoassay Calibrator

[0078] Calibration of an immunoassay is important for ensuring the quality of results generated in the immunoassay. Calibration generally involves the use of an immunoassay calibrator that contains the target analyte in a prescribed amount or concentration. The signal produced by the calibrator in an immunoassay is correlated to the amount of target analyte in the calibrator. This calibration, in turn, is used to correlate the amount of signal measured in a test sample with an amount of target analyte in the test sample. However, the signal generated by the calibrator may not represent the true amount of analyte in the calibrator if, for example, the target analyte in the calibrator is degraded or otherwise modified so as to corrupt the signal.

[0079] Accordingly, this invention provides methods for determining the quality of a troponin immunoassay calibrator. The method involves capturing molecules from an immunoassay calibrator used in an immunoassay against troponin with an antibody that captures troponin and specifically measuring the amount of troponin or one or more modified forms of troponin captured by the antibody. Alternatively, the immunoassay could be directed to measuring a particular modified form of troponin and involve the use of antibodies against this form and a calibrator that included this form.

[0080] The relative or absolute quantities of cardiac biomarkers and protein interactors with said biomarkers, in addition to clinical parameters such as patient signs and symptoms and electrocardiogram results, can be used for diagnosis, prognosis, and patient management purposes. For example, these results can diagnose the absence or presence of acute coronary syndrome as well as the specific class of acute coronary syndrome (*e.g.*, unstable angina versus recent myocardial infarction); determine the likely outcome of the patient in the absence of therapy (*i.e.*, determine prognosis), and determine whether the patient is likely to benefit from a course of specific medical therapy (*e.g.*, clotting inhibitors versus statins).

## VII. Determining the Quality of an Antibody in an Antibody Reagent Used in an Immunoassay

[0081] Immunoassays typically involve the use an immunoassay reagent that comprises an antibody directed against the target analyte. The accuracy of such assays depends upon the integrity and purity of the antibody in the immunoassay reagent. The presence of contaminants

in an antibody reagent can interfere with an accurate measurement of the amount of antibody in the antibody reagent. Accordingly, the present invention provides methods for determining the quality of an anti-troponin antibody used in an immunoassay reagent by specifically detecting modified forms of the antibody, *e.g.*, degraded forms, in the reagent.

**[0082]** In one version of the method, an anti-troponin antibody used in an immunoassay, in particular a commercial immunoassay, is examined by mass spectrometry. This analysis can indicate what portion of the antibody reagent is whole and what part is degraded. For example, the immunoglobulin may be degraded into heavy chains and light chains. Also, the immunoglobulin may be degraded into fragments of the heavy and light chains. Because mass spectrometry can distinguish intact immunoglobulin and degraded versions of it based on mass differences, the immunoglobulin reagent can thereby be qualified.

**[0083]** In another version of the method, the antibody is coupled to the surface of a SELDI probe and used to capture troponin from a sample or from a troponin calibrant for an immunoassay. This method can detect the absolute amount of intact troponin captured, as well as the relative amount of intact troponin to other molecules. The absolute quantity of an analyte as measured by an immunoassay is dependent on the quality of the reagents used to measure the analyte, as well as the quality of the reagents used to generate the standard curve (*i.e.*, the calibrators). If the antibody is not specific for the intended analyte, it may give false elevated levels. If the calibrator is impure, the calibration curve will be inaccurate. The inaccurate quantitation of an analyte can lead to the generation of incorrect conclusions regarding the optimal cutoffs for making medical decisions and can lead to the incorrect quantitation in individuals, leading to suboptimal management.

**[0084]** Having now generally described the invention, the same will be more readily understood through reference to the following exemplary embodiments, which are provided by way of illustration and are not intended to be limiting of the present invention unless specified.

## EXEMPLARY EMBODIMENTS

**[0085]** Anti-human cardiac troponin I antibodies (mouse monoclonal 8I-7), purified human troponin complex were obtained from Spectral Diagnostics, Inc. The antibodies were diluted to a final concentration of 0.5 mg/ml with 0.1M sodium bicarbonate 0.05% TritonX100 pH 9. Aliquots of 500 µl of antibodies were coupled to Reacti-gel (Pierce). The coupling was allowed to proceed at 4C for 16 hr. The beads were blocked with 1M TrisHCl pH 8 and then BSA (1mg/ml) in 0.5M TrisHCl, 0.1% TritonX100 pH 8. Excess antibodies were washed away

with 1% TritonX100 PBS, followed by 10% PEG 0.1% TritonX100 PBS and finally with 0.1% TritonX100 PBS.

**[0086]** Purified Troponin complex was diluted into 50% (v/v BSA 1mg/ml PBS) human serum (Intergen). Aliquots of 100 µl of each troponin complex standard were incubated with 4 µl of 50% slurry of anti-troponin antibodies immobilized on Reacti-gel. 50 µl of normal and cardiovascular disease patient sera (Genomics Collaborative, Inc) were diluted with 50 µl of BSA (1 mg/ml PBS, 0.1% Triton X100) and incubated with 4 µl of 50% slurry of anti-troponin antibodies immobilized on Reacti-gel. After 16 hr of incubation at 4°C with shaking, the gel beads were washed with 125 µl of 0.5% (w/v) polyethylene glycol 300, 0.05% Triton X100 PBS once, 125 µl of 1M urea 0.1% CHAPS 0.15M NaCl 50mM HEPES pH 7.5 once. An aliquot of beads was transferred to a hydrophobic ProteinChip® array (H4, Ciphergen), washed with 3 µl of 0.1M citric acid two times, and air dried. 2 µl of sinapinic acid were added per spot. The retained proteins were detected by PBSII mass spectrometer (Ciphergen). The molecules detected in this immunoassay are set forth in Table 1.

**[0087]** While specific examples have been provided, the above description is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of the specification. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

**[0088]** Although the foregoing invention has been described in detail by way of example for purposes of clarity of understanding, it will be apparent to the artisan that certain changes and modifications are comprehended by the disclosure and can be practiced without undue experimentation within the scope of the appended claims, which are presented by way of illustration not limitation.

**[0089]** All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted. By their citation of various references in this document, Applicants do not admit any particular reference is "prior art" to their invention.

Human Troponin C (SEQ ID NO:1)

MDDIYKAAVE QLTEEQKNEF KAAFDIFVLG AEDGCISTKE LGKVMRMLGQ NPTPEELQEM IDEVDEDGSG TVDFDEFLVM MVRCMKDDSK GKSEEELSDL FRMFDKNADG YIDLEELKIM LQATGETITE DDIEELMKDG DKNNDGRIDY DEFLEFMKGV E

Human Troponin I (SEQ ID NO:2)

MADGSSDAARE PRPAPAPIRR RSSNYRAYAT EPHAKKKSKI SASRKLQLKT LLLQIAKQEL EREAEERRGE KGRALSTRCQ PLELAGLGFA ELQDLCRQLH ARVDKVDEER YDIEAKVTKN ITEIADLTQK IFDLRGKFKR PTLRRVRISA DAMMQALLGA RAKESLDLRA HLKQVKKEDT EKENREVGDW RKNIDALSGM EGRKKKFES

Human Troponin T (SEQ ID NO:3)

MSDIEEVVEEY EEEEQEEAAV EEEEDWREDE DEQEEAAEED AEAEAETEET RAEEDEEEEE AKEAEDGPME ESKPKPRSFM PNLVPPKIPD GERVDFDDIH RKRMEKDLNE LQALIEAHFE NRKKEEEELV SLKDRIERRR AERAEQQRIR NEREKERQNR LAEERARREE EENRRKAEDE ARKKKALSNM MHFGGYIQKQ AQTERKSGKR QTEREKKKKI LAERRKVLAI DHLNEDQLRE KAKELWQSIY NLEAEKFDLQ EKFKQQKYEI NVLRNRINDN QKVSKTRGKA KVTGRWK